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(54) Title: USE OF INSULIN CONTAINING COMPOSITION FOR WOUND TREATMENT

(57) Abstract: Insulin provides reliable and effective prevention of scarring and/or at least a reduction in the severity of scarring. The application of insulin to wounds topically or by local injection is particularly advantageous since it simultaneously reduces/prevents scarring whilst enhancing re-epithelialisation of the wound and thus provides a dual action wound healing treatment. The present invention accordingly provides a highly effective prophylactic treatment for any individual suffering tissue trauma to reduce and/or prevent normal and/or pathological scarring.

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USE OF INSULIN CONTAINING COMPOSITION FOR WOUND TREATMENT

This invention relates to the pharmaceutical use of compositions for application to wounds. In particular,
5 the invention relates to the manufacture of a medicament for use in the treatment of scarring.

When injury, disease or surgery disrupts the normal architecture of body tissues such as the skin, eye and
10 palmar aponeurosis of the hand, the body instigates a complex cascade of events collectively known as wound healing. These processes in the early human foetus lead to total regeneration of the damaged or destroyed tissue. However, in post natal humans, although the
15 capacity of the outer layer of the skin, the epidermis, for regeneration is phenomenal, wound healing of the deeper skin layer, the dermis, is often accompanied by a fibroproliferative response that leads to the formation of a fibrotic scar. The tissues of the eye,
20 particularly the cornea, often scar after surgery or trauma which reduces the vision of the individual. Dupuytren's disease affects the palmar aponeurosis of the hand. This condition is caused by scarring and fibrosis that causes contraction of the palmar
25 structure, thus pulling the fingers permanently down towards the palm.

The severity of scarring of an individual in response to injury, disease or surgery is highly variable and
30 depends on multiple factors, such as infection, wound extent and orientation. Nevertheless, even when all these factors are taken into account, the severity of so-called "normal" scarring still varies dramatically between individuals. This variation in the severity of
35 scarring of the skin is perhaps most dramatically illustrated by the comparison of normal scars with

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pathological scarring conditions such as hypertrophic scarring.

Hypertrophic scars are characterised by extensive scar tissue, which contains an excess of collagen and is highly cellular (Rockwell et al., 1989. Plast. Recon. Surg. 84: 827 -837). Redness, hypopigmentation or hyper-pigmentation of the affected area often accompanies these scars. Patients can also suffer from hyperaesthesia and pruritus and, in addition, contraction of scars located over a joint can lead to a loss of mobility. This distressing pathological condition can affect substantial numbers of patients who have suffered various types of skin trauma, with children under the age of four years particularly prone. Two of the latest estimates of the proportion of paediatric burns patients who suffer from this condition are as high as 44 and 60%.

At present there is no method of predicting which individuals will develop these scars, nor any method of preventing their formation or that of normal scars, nor any treatment. Effective therapies for both pathological scarring and normal scarring accordingly continue to be sought.

It has been suggested for some time that insulin and its related growth factor family, insulin-like Growth Factors (hereinafter referred to as IGFs), may improve the re-epithelialisation of wounds. US 5591709 and US 5461030 of Life Medical Sciences Inc describe topically applied wound treatment formulations which are useful for treating wounds by accelerating wound healing. The formulations optionally contain insulin, together with further specified components. Although many groups (such as Pierre et al., 1998. J. Trauma. 44:34-345),

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have shown that systemic treatment with insulin and IGFs increases the speed of wound closure, the affect of these agents on scar tissue formation has never before been investigated.

5

To date, with regard to the development of anti-scarring therapies, attention has been focussed on the finding that transforming growth factor beta 1 (hereinafter referred to as TGF β 1) enhances scarring.

10

Many groups have worked to develop ways of inhibiting TGF β 1, with some success. Methods used have included TGF β 1 receptor blocking antibodies or the application of mannose-6-phosphate, which prevents the activation of latent TGF β 1. The main problem with these approaches 15 is that induction of scarring is not the only function of TGF β 1. TGF β 1 is known for its angiogenic properties. In addition, application of low doses of TGF β 1 is known to enhance re-epithelialisation of wounds. Thus, although the blocking of either the action or activation of

20

TGF β 1 in incisional wounds may result in the reduction of scarring with relatively few deleterious side effects, the healing of larger wounds, which rely on epithelial migration for closure, may be delayed.

25

We have now surprisingly shown that insulin prevents and/or reduces the formation of scar tissue whilst also improving the speed of re-epithelialisation of the wound. It is effective in preventing/reducing scarring of all types of wounds such as incisional wounds and 30 larger wounds.

35

According to a first aspect of the present invention there is provided the use of insulin for the manufacture of a medicament for reducing and/or preventing scarring.

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According to a second aspect of the present invention there is provided the use of insulin for the manufacture of a medicament for a dual action wound treatment comprising reducing and/or preventing scarring whilst accelerating and/or promoting wound healing.

According to a third aspect of the present invention there is provided a method of reducing and/or preventing scarring comprising administering a pharmaceutical composition comprising insulin to a wound.

We have now surprisingly found that insulin provides reliable and effective prevention of scarring and/or at least a reduction in the severity of scarring. The application of insulin to wounds topically or by local injection is particularly advantageous since it simultaneously reduces/prevents scarring whilst enhancing re-epithelialisation of the wound and thus provides a dual action wound healing treatment. The present invention accordingly provides a highly effective prophylactic and curative treatment for any individual suffering tissue trauma to reduce and/or prevent normal and/or pathological scarring.

Insulin

Insulin is a small (mw~6000) polypeptide hormone produced by the beta cells of the islets of Langerhans of the pancreas. It is made up of two chains of amino acids (designated A and B) which are held together by two disulphide bridges. Both chains are formed from the cleavage of a single helical chain known as pro-insulin, which consists of both the A and B chains of insulin connected by a peptide termed the C-peptide.

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Although the insulins of various species are highly homologous (differing only by a few amino acids) the sequence and number of amino acids making up the C-peptide can vary considerably. Under proper
5 conditions, three dimers of insulin associate to form a hexamer of appropriate dihedral symmetry that is stabilised by the presence of two zinc ions. Insulin affects cell metabolism via receptors present on the cell surface.

10

The term "insulin" as used herein includes within its scope a wide variety of insulin forms, and mixtures thereof. Suitable insulins are commercially available
15 from Hoechst, Lilly, Novo Nordisk and CP Pharmaceuticals, for example.

Insulins that are suitable for the uses in accordance with the present invention can be sourced from a
20 variety of different species due to the high degree of homology of insulin between species. Preferred insulins are those that are commonly available, including porcine, bovine or human insulins or mixtures thereof. The human insulins tend to be either derived
25 by enzymatic modification and purification from porcine insulin or originate from microorganisms using standard recombinant DNA technology techniques. Insulins that are suitable for the uses in accordance with the present invention include conventional insulins,
30 single-peak insulins, highly purified insulins, monocomponent insulins, purified insulins, human insulin (emp), semisynthetic insulins, human insulin (crb), human insulin (prb), human insulin (pyr) and biosynthetic human insulin. A wide variety of insulin forms are suitable for the uses of the present
35 invention for example, crystalline insulin, soluble

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insulin, neutral insulin, regular insulin and unmodified insulin, and formulations that prolong the duration of action of insulin such as suspensions formed by complexing insulin with a protein from which 5 it is slowly released (examples are "protamine zinc insulin" and "isophane insulin") or by modifying the particle size (e.g., insulin zinc suspensions) or biphasic insulins which are mixtures providing both immediate and prolonged action.

10 Chemical modifications of the insulin molecule has resulted in insulin such as delanated insulin (where the C-terminal alanine has been removed from the B chain of insulin), insulin defalan (where the terminal 15 phenylalanine has been removed), sulphated insulin, insulin argine and insulin lispro. Such chemically modified insulins are also suitable for the uses in accordance with the present invention. Furthermore, insulins obtained by standard recombinant DNA 20 technology are included within the scope of the present invention. Insulins obtained by standard recombinant DNA technology using nucleic acid chains that have a sequence identical to the naturally occurring gene encoding insulin in humans or other mammals are 25 preferred. This nucleic acid sequence may be modified by either conservative base substitutions, such that it encodes the same amino acid sequence of naturally occurring insulin; or modified with base substitutions which encode a different amino acid sequence from that 30 naturally occurring. Recombinant DNA technology has enabled production of other insulin analogues with altered pharmacokinetic profiles which are also included within the scope of the present invention.

35 The insulin to be employed in the uses according to the present invention is present in the pharmaceutical

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composition in an effective amount. Normally the total amount of the active is present in an amount between 50 picograms (1.25×10^{-6} IU) to 1000 micrograms (25IU) per millilitre of the composition. More preferably the 5 amount is 5 nanograms (1.25×10^{-4} IU) to 500 micrograms (12.5 IU) and most preferably from 50 nanograms (1.25×10^{-3} IU) to 50 micrograms (1.25IU), in order to maximise benefits at minimum cost.

10 Pharmaceutically Acceptable Vehicle

Most preferably the medicament in which the insulin is formulated for the uses according to the present invention is either a topical composition or an 15 injectable composition for injecting locally i.e. at or near to the site of the wound to be treated.

Topical and/or injectable compositions used according to the present invention also comprise a 20 pharmaceutically acceptable vehicle to act as a diluent, dispersant or carrier for the insulin. The vehicle may comprise materials commonly employed in wound treatment products such as water, saline solution, and, for topical compositions, liquid or 25 solid emollients, silicone oils, emulsifiers, solvents, humectants, thickeners, powders, propellents and the like.

The vehicle will usually form from 0.1% to 99.9%, 30 preferably 25% to 80% and can, in the absence of other adjuncts, form the balance of the composition.

In a particularly preferred embodiment the topical 35 composition comprises a delivery polymer that is saturated with the appropriate concentration of insulin.

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By "delivery polymers" is meant naturally occurring and/or synthetic polymers which facilitate the delivery of the active agent to its site of action. These polymers include hydrated or unhydrated hydrogels (e.g. 5 hydroxyethylmethacrylate (HEMA), glycerolmethacrylate (GMA) and polyvinylpyrrolidone (PVP); polyethylene glycol (PEG), methyl cellulose, agarose, extracellular matrix proteins such as collagens, fibronectins, fibrin, glycosaminoglycans, and mixtures thereof. In 10 general, 0.1 to 50% by weight of the composition of delivery polymer, is added to the insulin formulation to produce a gel.

Optional Adjuncts

15 Besides the insulin active, further adjuncts such as metal ions including zinc and chromium ions, anti-oxidants, antimicrobials, preservatives, opacifiers, colourants, perfumes, carrier proteins and buffers may 20 be present in the pharmaceutical composition.

Product Preparation, Form, Use and Packaging

To prepare the pharmaceutical composition to be used in 25 accordance with the present invention, the usual manner for preparing tissue treatment products may be employed. The active components are generally incorporated into a pharmaceutically acceptable vehicle in conventional manner. The active components can 30 suitably first be dissolved or dispersed in a portion of the water or other solvent or liquid to be incorporated in the composition. The preferred compositions are water/saline solutions of insulin.

35 The compositions may be in the form of conventional tissue treatment products such as cream, gel, lotions

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or solutions. The composition may be packaged in any suitable manner such as a jar, bottle, vial, tube or the like in a conventional manner.

5 The amount and frequency of application of the composition required for treatment will be readily apparent to one skilled in the art. In general, the treatment of a wound may be carried out by application, topically or by local injection, of a pharmaceutical
10 composition comprising the insulin to the wound one or more times daily. Typically, in solution or gel form, about 1ml of formulation is applied per cm² of the tissue trauma depending on the depth and severity of the wound to be treated. The extent of prevention
15 and/or reduction in scarring and enhanced re-epithelialisation of the tissue trauma will depend on the wound condition, the concentration of the active components, the amount of composition used and the frequency with which it is applied/injected.

20 In order that the present invention may be more readily understood, the following examples are given, by way of illustration only.

25 Example 1

One of the most important cell types in both normal and pathological scar formation is the myofibroblast. These cells, which differentiate from the unwounded tissue
30 cell type (fibroblasts), are responsible for laying down scar tissue. Indeed myofibroblasts remain present in hypertrophic scars up to four years after the original wounding event. An in vitro assay was accordingly developed to identify actives which prevent or reduce myofibroblast formation and thus identify
35 actives which are effective in reducing and/or

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preventing scar tissue formation.

Assay

5 Fibroblast cultures were initiated from normal skin, normal scars, hypertrophic scars (HTS) and burns scars.

Each culture was split into seven parts (A to G) and grown in the following different growth media:

10

(A) was grown in normal growth medium (hereinafter referred to as NGM) which consisted of Dulbecco's modified Eagles Medium (DMEM) plus 10% Foetal Calf Serum (FCS);

15

(B) was grown in growth factor depleted medium (hereinafter referred to as GF depleted) which is NGM that is depleted of active polypeptide growth factors including TGF β . It is prepared by treating FCS with a reducing agent in order to attack and cleave the disulphide bonds that determine the conformation and thus the biological activity of the polypeptide growth factors;

20

(C) (C_1-C_3) were grown in growth factor depleted medium plus 10ng/ml-5 μ g/ml of insulin (hereinafter referred to as GF depleted + I);

25

(D) (D_1-D_4) were grown in growth factor depleted medium plus 1ng/ml-100ng/ml of insulin - like Growth Factor - I (IGF-I) (hereinafter referred to as GF depleted + IGF-I);

30

(E) (E_1-E_3) were grown in growth factor depleted medium plus 50ng/ml - 200 mg/ml of insulin-like Growth Factor II (IGF-II) (hereinafter referred to as GF depleted + IGFII);

35

(F) (F_1F_3) were grown in growth factor depleted medium plus 25ng/ml to 100ng/ml of basic fibroblast growth factor (bFGF) (hereinafter referred to as GF depleted

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and bFGF);

(G) (G1-G3) were grown in growth factor depleted medium plus 25ng/ml to 100ng/ml of platelet derived growth factor (PDGF) (hereinafter referred to as GF depleted + PDGF).

5

The fibroblasts were grown in these media (which was replenished twice weekly) for fourteen days, fixed and stained immunohistochemically using an antibody specific for α -smooth muscle actin (a marker of myofibroblasts). The stain caused α -smooth muscle actin to fluoresce green and accordingly cells that had stained green were identified as myofibroblasts. The number of these was counted, as was the total number of cells present, and the proportion of myofibroblasts was then calculated. In this manner, the number of myofibroblasts present in each culture was determined as a percentage of the total number of cells in the culture population.

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ResultsTABLE 1

MEDIUM	Normal Skin	Normal Scar	HTS	Burns Scar
	NUMBER OF MYOFIBROBLASTS- PERCENTAGE OF TOTAL CELL POPULATION			
A - NGM	7.58	4.59	4.27	13.33
B - GF DEPLETED	19.15	34.71	35.14	42.55
C ₁ - GF DEPLETED + 10ng/ml INSULIN				16.44
C ₂ - GF DEPLETED + 100ng/ml INSULIN				15.91
C ₃ - GF DEPLETED + 5 μ g/ml INSULIN	5.26	6.14	3.23	8.20
D ₁ - GF DEPLETED + 1ng/ml IGF-I				40.79
D ₂ - GF DEPLETED + 10ng/ml IGF-I				43.90
D ₃ - GF DEPLETED + 50ng/ml IGF-I				51.51
D ₄ - GF DEPLETED + 100ng/ml IGF-I				47.00
E1 - GF DEPLETED MEDIUM + 50 ng/ml IGF-II				37.93

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	E2 - GF DEPLETED MEDIUM + 100ng/ml IGF-II				40.26
5	E3 - GF DEPLETED MEDIUM + 200ng/ml IGF-II				38.52
10	F1 - GF DEPLETED MEDIUM + 25ng/ml bFGF				31.82
15	F2 - GF DEPLETED MEDIUM + 100ng/ml bFGF				34.21
20	G1 - GF DEPLETED MEDIUM + 25NG/ml PDGF				50.95
25	G2 - GF DEPLETED MEDIUM + 50ng/ml PDGF				52.84
30	G3 - GF DEPLETED MEDIUM + 100ng/ml PDGF				53.35

35 Conclusions

A small but significant number of myofibroblasts are present in the NGM only cultures (see (A)). This phenomenon is thought to be due to the presence of TGF β in the FCS which makes up the medium, which is known to induce fibroblasts to differentiate into myofibroblasts whether they be derived from normal skin, normal scars or pathological scars.

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A surprisingly large number of myofibroblasts are present in the GF depleted cultures (see (B)). It was expected that because TGF β was thought responsible for the presence of the myofibroblasts in NGM cultures,
5 then the culture of fibroblasts in GF depleted medium should reduce the number of myofibroblasts seen in the population. However, contrary to expectation, no matter what the source of fibroblasts, culture in GF depleted medium promoted fibroblast differentiation into
10 myofibroblasts. This surprising result suggests that FCS contains actives that inhibit myofibroblast formation, with the biological activity of those actives being strictly dependant on the presence of intact disulphide bonds.

15 During attempts to identify the polypeptide constituent of FCS that was responsible for inhibition of myofibroblast differentiation, it was discovered that insulin but not its related factors IGF-I and IGF-II
20 nor bFGF or PDGF were capable of inhibiting myofibroblast differentiation. (Compare C with D to G Table 1). All the growth factors were titrated over their respective biofunctional ranges (insulin:
10ng/ml-5 μ g/ml, IGF-I:1-100 ng/ml IGF-II:50ng/ml-
25 200ng/ml, bFGF:25ng/ml - 100ng/ml, PDGF:25ng/ml - 100 ng/ml). All of these factors are known to be present in serum and their biological activities are strictly dependent on the presence of intact disulphide bonds.

30 Comparing B and C it can be seen that the addition of exogenous insulin specifically to fibroblasts cultured in GF depleted medium completely abrogates the induction of myofibroblast differentiation seen in this medium. Importantly, and further surprisingly,
35 comparing the data in C & D for burns scar cells it can be seen from the data presented in C that insulin is

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equally effective at inhibiting myofibroblasts differentiation for both normal skin, normal scar and pathological cells and thus is suitable both for preventing scar formation and reducing the extent of
5 scarring in both normal wound healing and pathological scarring conditions. Further surprisingly the related IGFs do not exhibit this technical effect thus demonstrating that the effect is insulin specific.

10 This finding, that the single specific growth factor insulin can prevent or at least reduce the formation of myofibroblasts, has wide implications in the fields of both cutaneous scarring and fibrosis generally. This new use of insulin as a treatment for preventing and/or
15 reducing scar tissue formation is accordingly an important medical advancement for tissue trauma sufferers. This new use is particularly beneficial as insulin also promotes the positive events of the wound healing process such as enhancing the re-
20 epithelialisation of wounds and thus a dual action treatment for wounds is accordingly provided for the first time.

EXAMPLE 2

25

In Vivo Study

The efficacy of an exemplifying treatment protocol using insulin in vivo was determined using a murine
30 incisional wound healing model in the manner described below.

After general anaesthesia, both posterior flanks on male BALB/c mice were shaved and cleaned with
35 chlorhexidine in spirit. Two 1.5cm wounds were marked (one in each flank) parallel to the spine with

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permanent marker. Full thickness incisions were made along these marks down to the level of the chest wall. Both lateral skin flaps were dissected from the underlying chest wall and the wound assessed from 5 haemorrhage. The wound edges were then infiltrated with test substance or vehicle only control, as appropriate, by injection into the wound edges. Insulatard® was diluted in normal saline (0.9%) to give a final test solution of 1 IU/ml. 0.15ml of this test 10 solution (0.15 IU) was applied to one of the test wounds and saline alone was applied to the contralateral wound on the same animal. Wounds were closed with interrupted 5/0 Prolene® suture. Mice were recovered from the anaesthetic individually and housed 15 in groups of 4. Wound healing was allowed to progress for 14 days (the time at which myofibroblast proportions are usually maximal), the mice were sacrificed and the wound area harvested for standard paraffin wax embedding and sectioning.

20 The wounds were analysed in the following manner: The comparison of the actual severity of scarring is impossible in this and all other animal models, since in contrast to human wounds, animal wounds are rapidly closed by way of swift epidermal contraction leaving 25 minimal areas of scarring in any case. We therefore attempted to judge the efficacy of insulin *in vivo* on its ability to affect the number of myofibroblasts present using immunohistochemical staining methods for the myofibroblast marker, α smooth muscle actin. To 30 allow semi-quantitative analysis of the resulting staining, a visual grading system was developed. Scars were assessed and allocated a score depending on the intensity of alpha smooth muscle actin staining within each section. The intensity of staining varied from 35 absent to strong and a numerical score was allocated to each staining intensity (i.e. 0 for absent staining, 1

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for weak, 2 for moderate and 3 for strong). Each scar was assessed at a number (minimum of 3) points along its length and the intensity was scored and averaged for each section to allow comparisons between treatments. Two independent assessors assessed the scoring blindly. The number of wounds assessed for each experimental group was 8. The results and statistical analysis are set out in Table 2 below:

10 TABLE 2

	Mean score of α - smooth muscle actin staining (out of maximum of 3)	Standard deviation	P Value (T Test)
Insulin	0.486	0.249	
Saline only	1.25	0.447	0.004

15

It can be seen from the data as presented in Table 2 that insulin is capable of significantly inhibiting myofibroblast differentiation in vivo.

20 EXAMPLE 3

25 The following injectable composition was prepared in conventional manner. It was found both to prevent and reduce scar tissue formation when locally injected into wounds in accordance with the present invention. The formulation is also suitable for topical application.

Each millilitre of the formulation contains:

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100IU of insulin (either human insulin (pyr) which is of recombinant origin produced in yeast), 3.78mg dibasic sodium phosphate, 1.76mg m-Cresol, 0.715mg phenol, zinc oxide, (content adjusted to provide 0.025 mg zinc ion), 0.28mg protamine sulphate, 16mg glycerin, and water to dilute to the required concentrations. The pH range of the composition is preferably 7.0-7.8, but 10% sodium hydroxide or hydrochloric acid may be used to adjust the pH, as required.

CLAIMS

1. Use of insulin for the manufacture of a medicament for reducing and/or preventing scarring.

5

2. Use of insulin for the manufacture of a medicament for a dual action wound treatment comprising reducing and/or preventing scarring whilst accelerating and/or promoting wound healing.

10

3. Use according to claim 1 or 2 wherein the medicament is a topical composition or an injectable composition.

15

4. Use according to claim 1, 2 or 3 wherein the insulin is present in an amount from 50 picograms (1.25×10^{-6} IU) to 1000 micrograms (25IU) per millilitre of the composition.

20

5. Use of insulin substantially as hereinbefore described with reference to Example 1, 2 and/or 3.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K38/28 A61P17/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 93 04691 A (LIFE MEDICAL SCIENCES INC.) 18 March 1993 (1993-03-18) claims 1-7, 10-21, 25-38 page 30, line 32 -page 31, line 8 ---	1-5
X	US 5 591 709 A (E. LINDENBAUM) 7 January 1997 (1997-01-07) claims 1-38 column 7, line 45 - line 60 column 13, line 15 - line 23 ---	1-5

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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WO 9304691	A	18-03-1993	AT AU AU BR CA DE DE EP FI HU JP NO WO US US	186840 T 670413 B2 2587992 A 9206433 A 2116549 A1 69230344 D1 69230344 T2 0650366 A1 940932 A 67319 A2 6510453 T 940406 A 9304691 A1 5461030 A 5591709 A		15-12-1999 18-07-1996 05-04-1993 27-09-1994 18-03-1993 30-12-1999 13-04-2000 03-05-1995 28-02-1994 28-03-1995 24-11-1994 28-03-1994 18-03-1993 24-10-1995 07-01-1997
US 5591709	A	07-01-1997	US AT AU AU BR CA DE DE EP FI HU JP NO WO	5461030 A 186840 T 670413 B2 2587992 A 9206433 A 2116549 A1 69230344 D1 69230344 T2 0650366 A1 940932 A 67319 A2 6510453 T 940406 A 9304691 A1		24-10-1995 15-12-1999 18-07-1996 05-04-1993 27-09-1994 18-03-1993 30-12-1999 13-04-2000 03-05-1995 28-02-1994 28-03-1995 24-11-1994 28-03-1994 18-03-1993
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